Journal of the Minnesota Academy of Science

Volume 31 | Number 1 Article 14

1963

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Introduction. The detection of a circadian (about 24-hour) susceptibility-resistance cycle to acetylcholine by spotchecks on mortality from this agent will be here presented. The study was done under conditions standardized to some extent for 24-hour routine-synchronized circadian system analysis (1). Sampling was limited to only two time points along the 24-hour scale. Hence, results represent a spotcheck rather than a circadian map. The conclusions to be drawn from these data have been confirmed, in the interim, with more frequent sampling. The data to be reported constitute further evidence that circadian system structure (2) involves significant and predictable changes in the reactivity of organisms to various agents.

Materials and methods. The mice used were all mature animals maintained in the Department of Pathology at the University of Minnesota. The several strains tested had been brother-to-sister mated for over 10 years.

The animals were transferred to a sound-deadened periodicity room at the Cambridge State School and Hospital and were there standardized 5 mice/cage for over one month. The room was kept at 24 ± .9° C and illuminated by artificial light only, daily from 0600 to 1800. Since weaning, during standardization and throughout the sampling period, food and water were available to the animals *ad libitum.*

Acetylcholine was injected intraperitoneally. Doses were adjusted to 20 gm. of body weight. Mortality was checked continuously for the first hour post-injection. Thereafter the mice were checked daily for the next 7 days. Most deaths occurred within 25 minutes after injection.

Results. A first single-dose experiment was done on a total of 300 animals from the inbred C (Bagg albino), D_8 (Dilute Brown, subline 8), and B_1 (C_{57} Black, subline 1) strains. Mice of both sexes showed a higher mortality at 2000 as compared to 0800, the difference being statistically significant, as may be seen from Table 1.

In a second study, two dose levels were tested (at each of two test times) on two groups, each of 30 B_6 (C_{57} Black, subline 6) male mice. With the more limited number of mice in this experiment, results with the lower dose level tested were not statistically significant. But again the percentage of deaths from injections at 2000 was higher than that from injections at 0800. The same applies to results on the mice receiving a slightly higher dose level of acetylcholine, the within-day difference in mortality being significant below the 5% level in these samples.

TABLE 1. Spotcheck on Circadian Susceptibility-Resistance Cycle to Acetylcholine of Inbred C, D_8 and B_1 Mice of both Sexes and of Male B_6 Mice as a Function of Circadian System Phase.*

Exp. No.	Mouse-Strains Tested	Dose mg/20 gm body wt.	Test-Time	Total No. of Mice	No. of Deaths	%	X^2	P
1	C, D_8, B_1	4	0800	151	54	35.8	10.6	.001
			2000	149	105	70.5		
2a	B_6	4.0	0800	15	5	33.3	0.5	.44
			2000	15	8	53.3		
2b	B_6	4.4	0800	15	6	40.0	5.2	.025
			2000	15	13	86.6		

* Light from 06–18, alternating with darkness.

The data as a whole demonstrate a circadian susceptibility-resistance cycle. They also suggest that under the conditions of such experiments more than 15 mice/time-point are necessary for the consistent demonstration of susceptibility-resistance cycles (3), such as that here disclosed for the reactivity of mice to acetylcholine.

Added studies now under evaluation done at 4-hour intervals on B_6 male mice definitively demonstrate statistically significant circadian changes along the 24-hour scale in mortality from acetylcholine and prompt, further, a study of underlying mechanisms, with a view of endpoints of drug effect less drastic than death.

Summary and conclusions. It seems well established for acetylcholine and for a number of other agents that the endpoint death can be a predictable function of circadian system phase (4-13). Observations made simply as a function of time of day also are pertinent in this connection (14, 15), but their reproducibility from one study to the next will depend heavily upon the extent of standardization of conditions of observation and sampling (1, 3). In the absence of such standardization, in studies done as a function of time of day without further qualification contradictory results can be expected (16, 17), as has been emphasized elsewhere (1). Analytical statistical considerations are pertinent in the same connection (3).

* Supported by grants from the United States Public Health Service (NB-04531, C-4359) and the State of Minnesota. Thanks for valuable assistance in these studies also are due to Mrs. Frank Jones and Mrs. Marjorie Lindgren. Dr. Robert H. Silber, Associate Director, Merck Institute for Therapeutic Research, provided invaluable help to these and related studies.

* Received for publication, August 1, 1963.

In any event, definition of timing for animals or tissues used in many assays done *in vivo* (18-22) or *in vitro* (23, 24) seems mandatory, if excessive variability of test results is to be avoided. Moreover, detection of spontaneous circadian susceptibility-resistance cycles facilitates studies of possible underlying cyclic factors that can be analyzed by the comparative study of shift-times and other procedures derived from circadian system analysis (25).

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